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Characterization of fungicide-resistant isolates of *Penicillium digitatum* collected in California

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Abstract

Isolates of *Penicillium digitatum*, cause of citrus green mould, were collected in California from infected fruit from packing houses or groves. The fungicide sensitivity of 166 isolates to imazalil (IMZ), thiabendazole (TBZ), sodium *ortho*-phenylphenate (SOPP), and pyrimethanil (PYR) were determined. All of these fungicides except PYR were in use in packing houses. None were used in groves. Isolates resistant to IMZ, TBZ, and SOPP occurred within packing houses but not in groves. Many were simultaneously resistant to two or more fungicides. Resistance to PYR was found only in three isolates from relatively isolated groves in northern California. The EC_{50} levels of IMZ and SOPP among resistant isolates varied, while those resistant to TBZ were primarily of one level. The colony colour, lesion expansion rate and days to sporulate on infected lemons, and the magnitude of sporulation were determined for many isolates. Some minor alterations in colony colour and a slightly reduced lesion size occurred among fungicide-resistant isolates, particularly those resistant to more than one fungicide. Lemons were inoculated with a mixture of conidia from one sensitive and one resistant isolate in equal portions, and then conidia were collected one week later from lesions. The resistant isolates were all resistant to IMZ and some were also resistant to SOPP and TBZ. The proportion of IMZ-sensitive and -resistant conidia was determined and comprised the inoculum to initiate a subsequent decay cycle. A total of 28 pairs of sensitive and resistant isolates were evaluated over four cycles. IMZ-resistant conidia declined rapidly in 26 pairs; few or no IMZ-resistant conidia were present after four cycles. In two pairs the resistant conidia persisted over four cycles with little decline, which suggests that in the absence of IMZ use some resistant isolates may persist for long periods. All of the fungicides would effectively control green mould on fruit arriving from groves with incipient infections, because sensitive isolates predominate there, however, control of infections initiated within packing houses, where resistant isolates predominate, remains a difficult problem. The recently introduced PYR controls resistant isolates that now occur in packing houses, but resistance to this fungicide, which was detected in three isolates from locations where PYR had not been used, indicates it must be used with good resistance management practices.
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1. Introduction

Green mould, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc., is the most important post-harvest disease of citrus fruits in California (Holmes and Eckert, 1999). Sodium *ortho*-phenylphenate (SOPP), thiabendazole (TBZ), and imazalil (IMZ) are commonly used fungicides in California packing houses. Each with a different mode of action, they are used alone, combined in mixtures, or applied separately in sequence, and they have been the primary method used to control citrus fruit decay during storage and marketing for more than 25 years (Ismail and Zhang, 2004). Factors that contribute to the proliferation of fungicide-resistant isolates within packing houses include the prolonged and occasional excessive use of the same fungicides in

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combination with the year round operation of packing houses without a pause to conduct sanitation measures (Holmes and Eckert, 1999). Brown and Miller (1999) and Holmes and Eckert (1995) stated that discriminatory concentrations used to classify isolates of *P. digitatum* resistant to IMZ, TBZ, or SOPP were 0.1, 10.0, or 15.0 $\mu g\,ml^{-1}$, respectively. If *P. digitatum* is capable of growth on agar media amended with these discriminatory fungicide concentrations, it will be poorly controlled by these fungicides applied commercially to citrus fruit at approved rates. Resistant isolates to each of these widely used fungicides were reported in citrus packing houses in California (Eckert, 1987, 1990) and elsewhere (Harding, 1972; Dave et al., 1989; Bus et al., 1991; Bus, 1992). A reduction in IMZ efficiency caused by IMZ-resistant isolates was demonstrated in commercial citrus packing houses (Eckert et al., 1994). The mode of action of IMZ is the inhibition of cytochrome p450 and resistance among isolates of *P. digitatum* was associated with altered regulatory regions that affected over-expression of cytochrome p450 synthesis (Hamamoto et al., 2000).

SOPP, IMZ, and TBZ are effective enough to remain in use, primarily because resistant isolates are rare within groves, so they effectively control infections initiated in groves on fruit entering the packing house. Actions in the packing houses, such as heating the fungicide solution or adding $NaHCO_3$, have been introduced to improve the effectiveness of the fungicides so that some control of resistant isolates occurs (Smilanick et al., 2005, 2006). Measures that improve sanitation in packing houses can reduce the proliferation of resistant isolates (Hall and Bice, 1977; Bancroft et al., 1984; Gardner et al., 1986). A common practice that provides advance warning of a developing resistance problem is the periodic monitoring within packing houses accomplished by the exposure of fungicide-amended media to collect airborne conidia (Houck, 1977; Förster et al., 2004). When resistance to a fungicide is detected, an alternate fungicide with a different mode of action should be used. Recently, two new post-harvest fungicides, pyrimethanil (PYR) and fludioxonil, were introduced into the citrus industry, primarily to use in alternating strategies with the existing fungicides (Goodwine, 2005; Smilanick et al., 2006).

Resistance to SOPP was first reported among *P. digitatum* isolates from California citrus packing houses within a few years of its introduction, more than 50 years ago (Harding, 1962). Resistance to TBZ occurred about 2 years after its introduction in 1968 (Harding, 1972). Resistance to IMZ developed more slowly and was first noted among *P. digitatum* isolates collected in California citrus packing houses in 1986, about 6 years after it was introduced (Eckert, 1990). Van Gestel (1988) showed isolates "less sensitive" to IMZ did not compete fully with the sensitive isolates. Holmes and Eckert (1995) reported fungicide resistance to IMZ, TBZ, or both was stable after many transfers of resistant isolates to fruit or media in the presence or absence of the fungicide. Resistance did not influence growth characteristics of the pathogen, and that in mixtures with sensitive isolates inoculated into fruit or on growth media resistant isolates would decline markedly after several cycles if the fungicide was not present. Earlier work by Seidel et al. (1990) that characterized TBZ-resistant isolates had similar conclusions. Fungicide resistance has remained a serious problem for the citrus industry and characterization of these isolates remains important, particularly if some aspect of their fitness has changed and they have become more competitive.

The purpose of this study was to determine if changes in the distribution or characteristics of fungicide-resistant populations of *P. digitatum* had occurred and to establish the baseline sensitivity of this pathogen to a new fungicide, PYR. Specific objectives were to collect isolates from packing houses, individual trees, and citrus groves in central California that were resistant (R) or sensitive (S) to the fungicides now in use, to characterize growth rate, virulence, and colony colour of these isolates, and to evaluate their competitiveness in a mixed population on non-treated citrus fruit. In addition, the sensitivity of many isolates to PYR was determined, collected before it was approved for post-harvest use on citrus fruit in California.

2. Materials and methods

2.1. Origin and storage of *P. digitatum* isolates

To determine the distribution of fungicide resistant isolates, *P. digitatum* from the primary citrus production areas in California was collected during 2004 and 2005. It was isolated from orange or lemon fruit with green mould symptoms that were located in groves (25), packing houses (22), or under individual trees located in central California (San Joaquin Valley and Pacific coastal areas). A sterile wooden toothpick was rubbed on sporulating areas of lesions on infected fruit and then its tip was broken off inside a sterile microcentrifuge tube (1.5 ml capacity) that contained 1 ml of an aqueous solution of 0.01% Triton X-100 (w/v) (Sigma-Aldrich, St. Louis, MO, USA). Tubes were vortexed 4–5 s to suspend the conidia. This suspension of conidia was streaked onto potato-dextrose agar (PDA; Difco Laboratory, Detroit, MI, USA) in a 9-cm diameter Petri dish. Plates were kept at 25 °C for 24 h, and then a single hyphal tip was excised with a scalpel and cultured on fresh PDA plate. After growth for 1 week at 25 °C, conidia were collected and stored on silica gel at −40 °C (Perkins, 1962).

2.2. Determination of fungicide sensitivity

An EC_{50} value, defined as the concentration of a fungicide that inhibited colony diameter on PDA by 50%, was determined for 166 isolates using IMZ (Fungafluor 50, 44.6% w/v, Janssen Pharmeceutica, Beerse, Belgium), TBZ (Sealbrite, 99.0% w/v, EcoScience Corp., Orlando, FL, USA), SOPP (Freshgard 20, 23% w/v, FMC

Corp., Riverside, CA, USA), and PYR (PenbotectM 400 SC, 40% w/v, Janssen Pharmaceutica, Beerse, Belgium). The pH of the medium was 5.6 ± 0.2 in all tests. Conidia of a 1-week-old culture grown on PDA of each isolate were collected in 5 ml of 0.01% Triton X-100, filtered through two layers of cheesecloth, transferred into sterile flask, and the conidial suspension was adjusted with sterile water to an optical density of 0.1 at 425 nm using a spectrophotometer, which contained about 10^6 conidia ml^{-1} (Eckert and Brown, 1986). A 10 μl droplet of the conidial suspension was placed on PDA inside a 90 mm diameter Petri dish. Six isolates per plate were evaluated. The media were amended with one of the fungicides at the following concentrations (μg ml^{-1}): (a) IMZ at 0.05, 0.1, 0.5, 1, or 2.5; (b) TBZ at 0.05, 0.1, 1, 10, or 25; (c) SOPP at 5, 10, 20, 40, or 60; or (d) PYR at 0.05, 0.1, 0.5, 1, or 2.5. Non-amended PDA was used to grow all isolates as a control. All the cultures were repeatedly examined, and the time (in d) was recorded when sporulation began. After incubation for 4 d at 25 °C, colony diameters were measured and the inhibition in growth was calculated. The experiment was done three times.

2.3. Isolate characteristics

The colour of colonies on PDA, the size of lesions they caused on lemons, and time of initiation of sporulation from these lesions was determined for *P. digitatum* isolates. Three replicates of each of 182 isolates were cultured for 10 d at 24 °C with 12 h cool white fluorescent light daily, on PDA, then the colony colour was determined with a colourimeter that used the CIElab system of colour notation (model CR200, Minolta Corp., Tokyo, Japan). L^*, a^*, and b^* colour space values were recorded and hue angle was calculated for each isolate (McGuire, 1992). The colourimeter has an internal pulsed xenon arc lamp light source (average sunlight or CIE Standard Illuminant C) that illuminates 8 mm of the sample surface at a 0° viewing angle for each measurement. The experiment was repeated twice. To determine when sporulation occurred and record lesion size, 67 isolates were similarly cultured on PDA, their conidia were collected in sterile water and adjusted with sterile water to an optical density of 0.1 at 425 nm using a spectrophotometer, which contained about 10^6 conidia ml^{-1} (Eckert and Brown, 1986). Five replicate lemon fruits were inoculated with each isolate by a single injection of each fruit with 0.2 ml at a depth of 1 cm below the rind surface. After inoculation, the fruits were placed at 20 °C. The fruits were repeatedly examined, and the time (in d) was recorded when sporulation began. After 7 d, the diameter of the decay lesions and the magnitude of sporulation were recorded. Sporulation was recorded using a visual index, where 0 = no sporulation or surface mycelia was present; 0.5 = surface mycelia growth; 1 = some sporulation present to 5% of the surface area; 2 = 6–20%; 3 = 21–60%; 4 = 61–90%; and 5 = 91% or more. The experiment was done twice.

2.4. Competition among isolates on fruit

IMZ-sensitive (S; $n = 6$) and IMZ-resistant (R; $n = 10$) isolates were selected for competition studies. They were selected because they represented a geographically diverse sample of the isolates collected. All of the S isolates were from infected fruit found in groves or under isolated trees, while R isolates were from infected fruit in packing houses. Isolates from silica gel were re-cultured on PDA for 7 d at 20 °C, a small volume of sterile Triton X-100 (0.05% w/v) was added, the surface of the colony was rubbed with a sterile glass rod, and the solution passed through two layers of cheesecloth. The spore concentration was adjusted with sterile water to an optical density of 0.1 at 425 nm using spectrophotometer, which contained about 10^6 conidia ml^{-1} (Eckert and Brown, 1986). Spore suspensions of S and R isolates were mixed in a 1:1 ratio (10^6 conidia ml^{-1}). Twenty-eight unique combinations were prepared. For each combination of S and R isolates, three replicate Valencia oranges were washed with 200 μg ml^{-1} chlorine, a single puncture 2 mm deep and 1 mm wide was made with a sterile steel tool, and 20 μl of the spore suspension was placed into each wound. After inoculation, fruit were placed inside plastic boxes and incubated at 20 °C, with a high relative humidity maintained by the placement of a moistened paper sheet within each box. Seven days later, several milligrams of dry conidia were aseptically collected with a sterile scalpel within an area of several centimeters around the wound site, placed in the Erlenmeyer flasks containing 10 ml of Triton X-100 (0.05% w/v), and adjusted to 10^6 conidia ml^{-1}. The next decay cycle was initiated immediately by mixing equal portions of the adjusted spore suspensions from three replications and inoculating three fruit as previously described. The proportion of IMZ-sensitive and -resistant conidia was determined by dilution of the inoculum mixture with sterile water to a concentration of 10^3 conidia ml^{-1} and application of 200 μl of this suspension to three Petri dishes containing dichloran rose Bengal chloramphenicol agar (DRBC; EM Science, Gibbstown, NJ, USA), alone or amended with 0.1 μg IMZ ml^{-1}. After about 5 d of incubation at 20 °C, the numbers of colonies were enumerated and proportion of IMZ-resistant conidia recorded. The proportion of S and R conidia was determined in the initial inoculum and for four decay cycles.

2.5. Statistics

All analyses were conducted using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). The EC_{50} of the isolates, defined as the concentration of the fungicide that caused a reduction in colony diameter by 50%, was estimated by linear regression of the log of the colony diameter versus the fungicide concentration (Mondal et al., 2005). Colour values, colony and lesion

sizes, and the interval between inoculation and sporulation on lesions were analysed by ANOVA followed by Fisher's protected LSD ($P<0.05$).

3. Results

3.1. Determination of fungicide sensitivity

Resistance to IMZ, TBZ, and SOPP was common among the 120 isolates collected from packing houses but absent among the 46 isolates from groves or trees (Fig. 1 and Table 1). The frequency of the occurrence of resistant isolates and the level of fungicide resistance among packing house isolates was high. Among the 120 isolates from packing houses, the EC_{50} of 86% of them was 0.5 μg ml^{-1} IMZ or more, the EC_{50} of 48% of them was 10.0 μg ml^{-1} TBZ or more, and the EC_{50} of 30% of them was 15.0 μg ml^{-1} SOPP or more. Resistant isolates were often resistant to two or more fungicides simultaneously. Among the packing house isolates, 13 were sensitive to all of the fungicides, three were resistant to TBZ alone, 56 were resistant to IMZ alone, 20 were resistant to both IMZ and TBZ, and 27 were resistant to IMZ, TBZ, and SOPP.

The EC_{50} of PYR to isolates among both grove and packing house isolates was a relatively low concentration and within a narrow range with three exceptions (Fig. 1 and Table 1). PYR was not used on citrus fruit, either before or after harvest, in California at the time the isolates were collected. The EC_{50} of three isolates (about 5% of those examined) collected from relatively isolated groves in northern California were five- to ten-fold higher than others.

3.2. Colony characteristics

The fungicide resistance of isolates was associated with very small but significant colour changes expressed as L and hue angle (Table 2) compared to fungicide-sensitive isolates. The L of isolates resistant to IMZ, TBZ, and SOPP was significantly higher than the other isolates, an indication of a lighter colony colour. The hue of isolates was significantly lower (an indication of less green and increased yellow colour) among isolates resistant to both IMZ and TBZ, while the hue angle was greater (an indication of increased green colour) among isolates resistant to only IMZ.

Sporulation of colonies of isolates resistant to the fungicides was delayed or prevented, particularly with SOPP, when they were cultured on fungicide-amended PDA. Sporulation was visible on colonies of isolates of *P. digitatum* cultured on unamended PDA between the second and third day. Of 39 isolates resistant to SOPP cultured on PDA amended with 20 μg ml^{-1} SOPP, although colony growth was evident, sporulation of 23 of them was prevented and that of 16 was delayed and appeared after 5–6 d. On PDA amended with 1 μg ml^{-1} of IMZ or 10 μg ml^{-1} of TBZ, sporulation of isolates capable of growth on these fungicides was not stopped but delayed and appeared after 5–6 d. Sporulation of the three PYR-resistant isolates cultured on PDA amended with 2.5 μg ml^{-1} of PYR was delayed 2 d compared to their sporulation on PDA alone.

The period between inoculation and the first appearance of conidia (d to sporulation) and the abundance of sporulation on decay lesions on fruit did not differ significantly between IMZ, TBZ, or SOPP-sensitive and -resistant isolates (Table 3). The diameter of decay lesions caused by the sensitive isolates was significantly larger than

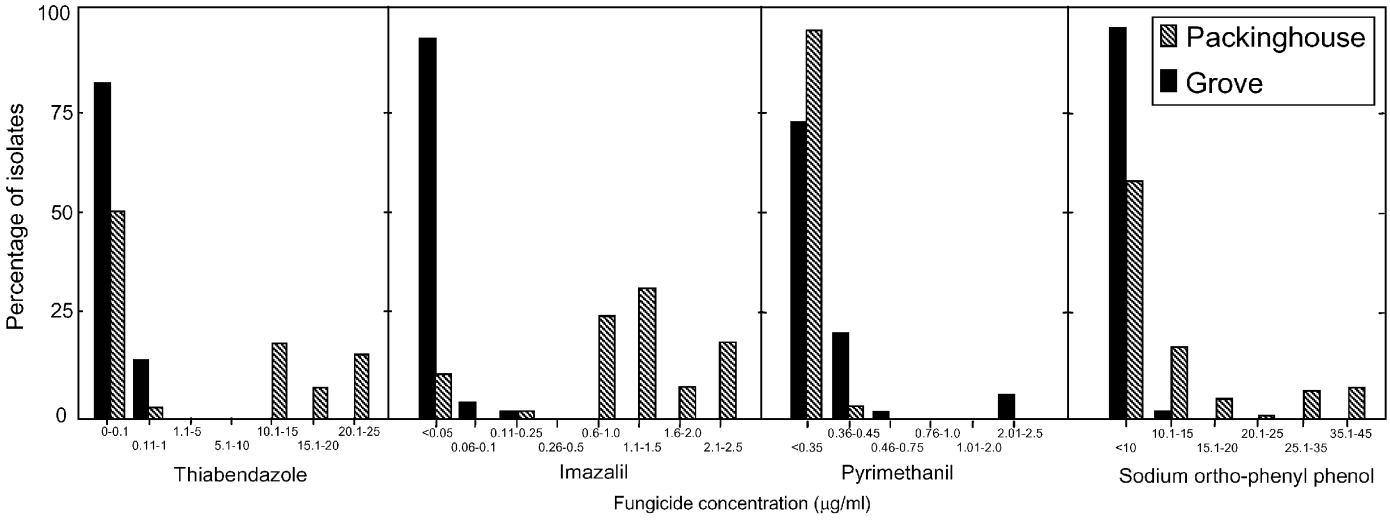

Fig. 1. Classification of the fungicide sensitivity of 166 *Penicillium digitatum* isolates to thiabendazole, imazalil, pyrimethanil, and sodium *ortho*-phenylphenate collected in California groves and packing houses. Isolates were classified by their EC_{50} values which were the concentration of the fungicide that reduced colony diameter on fungicide amended potato-dextrose agar by 50% compared to that on potato-dextrose agar alone.

Table 1
The mean concentrations of imazalil (IMZ), thiabendazole (TBZ), and sodium *ortho*-phenyl phenate (SOPP) that inhibited the size of colonies of *Penicillium digitatum* on potato-dextrose agar by 50% (EC_{50})

Fungicide	EC_{50} ($\mu g\,ml^{-1}$) ($\pm$SD)			
	Sensitive	n[a]	Resistant	n
IMZ	0.044 ($\pm$0.023)	66	1.45 ($\pm$0.58)	100
TBZ	0.10 ($\pm$0.10)	113	17.7 ($\pm$4.8)	53
SOPP	8.8 ($\pm$1.7)	137	30.1 ($\pm$9.1)	29
PYR	0.30 ($\pm$0.07)	163	2.39 ($\pm$0.19)	3

Isolates were classified as sensitive or resistant to each fungicide.
[a]n = Number of isolates of *P. digitatum*.

Table 2
The colour of colonies of isolates of *Penicillium digitatum* cultured for 10 d on potato-dextrose agar at 24 °C with 12 h cool white fluorescent light, that were resistant to imazalil (IMZ) alone, to both IMZ and thiabendazole (TBZ), or to IMZ, TBZ, and sodium *ortho*-phenylphenate (SOPP), or sensitive to all three

Isolate	n	Hue angle[a]	L^*[b]
Sensitive	50	93.3 b[c]	22.7 a
IMZr	56	94.1 c	20.7 a
IMZr + TBZr	20	90.6 a	23.8 ab
IMZr + TBZr + SOPPr	27	93.1 b	24.9 b
P		0.0001	0.0001

The EC_{50} of isolates resistant to IMZ, TBZ, and SOPP equals or exceeds 0.5, 10, and 15 $\mu g\,ml^{-1}$, respectively.
[a]Hue angle expressed in degrees, higher or lower values within those shown indicate trends to green or yellow, respectively.
[b]L^* indicates black to white intensity in a scale of 0–100, higher values are lighter.
[c]Values within columns followed by unlike letters are significantly different by Fisher's Protected Least Significant Difference test.

those caused by isolates simultaneously resistant to IMZ, TBZ, and SOPP. Lesions caused by the other resistant isolates were not significantly smaller than those caused by the sensitive isolates.

3.3. Competition among isolates during repeated infection cycles on oranges

The origin of the isolates used in competition experiments, their EC_{50} concentrations of IMZ, TBZ, SOPP, and PYR, and the size of lesions they caused on lemons are shown (Table 4). Lesions caused by isolates sensitive to the fungicides were typically larger than those caused by resistant isolates; often these differences were significant. The EC_{50} concentrations of PYR of the isolates were 0.22–0.52 $\mu g\,ml^{-1}$. The proportion of IMZ-resistant conidia was determined in a total of 28 combinations of the S and R isolates over four disease cycles on fruit (Fig. 2). The proportion of conidia of the IMZ-resistant isolates declined rapidly in 26 combinations, where typically few or none of the resistant conidia were present after four cycles of inoculation. However, in two combinations of IMZ-resistant and -sensitive conidia (combination S9 and R33 and combination S21 and R45), the proportion of IMZ-resistant conidia began to decline after two or three cycles and was still relatively high after four cycles.

4. Discussion

To manage fungicide-resistant pathogens effectively, it is important to monitor the occurrence and distribution of resistant isolates, as well as the level of resistance to the fungicide applied (Brent, 1988). In our survey, isolates resistant to IMZ, TBZ, and SOPP were common among those collected from packing houses, where post-harvest fungicides are frequently used. Many isolates were resistant

Table 3
Characteristics of lesions on lemon fruit caused by isolates of *Penicillium digitatum* that were resistant to imazalil (IMZ) alone, thiabendazole (TBZ) alone, to both IMZ and thiabendazole (TBZ), to IMZ, TBZ, and sodium *ortho*-phenylphenate (SOPP), or sensitive to all three

Isolate	n[a]	Days to sporulation	Sporulation[b]	Lesion diameter (mm)
Sensitive	12	3.4	3.6	33.2 a[c]
IMZr	42	3.7	3.4	29.2 ab
TBZr	2	3.4	3.5	29.0 ab
IMZr + TBZr	1	3.8	2.9	28.9 ab
IMZr + TBZr + SOPPr	6	3.4	3.4	28.1 b
P		NSD	NSD	0.0001

The period between inoculation and the appearance of conidia on decay lesions (days to sporulate), the abundance of conidia on sporulating lesions after 7 d, and the diameter of decay lesions after 7 d were recorded.
[a]n = number of isolates of *P. digitatum*.
[b]Sporulation was recorded using a visual index, where 0 = no sporulation or surface mycelial growth was present; 0.5 = surface mycelial growth present; 1 = some sporulation present to 5% of the surface area; 2 = 6–20%; 3 = 21–60%; 4 = 61–90%; and 5 = 91% or more.
[c]Values within columns followed by unlike letters are significantly different by Fisher's Protected Least Significant Difference test. NSD indicates no significant differences were present.

Table 4
Characteristics of isolates of *Penicillium digitatum* used in competition experiments including isolate origin, EC_{50} concentrations of the fungicides imazalil (IMZ), pyrimethanil (PYR), sodium *ortho*-phenylphenate (SOPP), and thiabendazole (TBZ) on amended potato-dextrose agar, and lesion diameter on fruit after 5 d at 20 °C

Isolate	Origin[a]	Resistance	Fungicide EC_{50} ($\mu g\,ml^{-1}$)				Lesion diameter[b]
			IMZ	PYR	SOPP	TBZ	
S9	A	None	0.04	0.22	8.67	0.07	35.8±4.6 ab
S10	A	None	0.07	0.23	9.00	0.07	37.4±5.2 ab
S21	A	None	0.04	0.30	8.33	0.07	39.2±1.9 a
S24	A	None	0.05	0.26	8.33	0.07	33.8±1.9 abc
S27	A	None	0.03	0.52	8.67	0.07	35.0±4.8 abc
S40	A	None	0.03	0.25	5.50	0.08	33.2±4.6 bc
R5	B	IMZ	1.03	0.32	9.67	0.07	26.8±3.6 de
R30	B	IMZ	1.40	0.25	7.00	0.08	30.7±4.7 cd
R57	B	IMZ	1.25	0.25	6.00	0.08	29.1±5.2 de
R66	B	IMZ	2.00	0.25	9.00	0.08	27.0±3.4 de
R33	B	IMZ/TBZ	1.75	0.25	7.50	25.00	26.6±3.9 e
R54	B	IMZ/TBZ	1.75	0.25	7.50	22.50	28.9±4.5 de
R38	B	IMZ/TBZ/SOPP	1.05	0.25	29.00	12.00	26.9±5.0 de
R44	B	IMZ/TBZ/SOPP	0.95	0.25	27.50	15.00	27.3±3.6 de
R45	B	IMZ/TBZ/SOPP	1.50	0.25	15.00	25.00	27.2±2.4 de
R46	B	IMZ/TBZ/SOPP	1.15	0.25	40.00	17.50	28.5±4.2 ab

[a] *Origin*: A = isolated from decayed fruit from within citrus groves or under single trees with no history of fungicide use and not located near citrus packing houses; B = isolated from decayed fruit located within citrus packing houses where these fungicides were in use or recently in use at the time of the collection.

[b] Lesion diameter (+SD) in mm on lemons after 3 d at 20 °C. Each value is the mean of three experiments of five fruits, each with one lesion. Values followed by unlike letters differ significantly by Fisher's Protected Least Significant Difference test ($P \leqslant 0.05$).

to two or more fungicides simultaneously. Conversely, resistant isolates were not found in groves, where fungicides are not used. These results are in agreement with many earlier studies of *P. digitatum* fungicide resistance in California and elsewhere (Harding, 1972; Houck, 1977; Holmes and Eckert, 1995). We found fewer isolates that were resistant solely to TBZ compared to those resistant to either IMZ alone or simultaneously to both TBZ and IMZ. Most packing houses constantly use IMZ and add TBZ periodically, typically when IMZ resistance becomes a problem. Presumably, within those periods where TBZ is not used, there is some decline in TBZ-resistant isolates in the packing house. SOPP use in citrus packing houses has declined and became rare in recent years. Although we found SOPP resistant isolates in packing houses, this resistance was always accompanied by resistance to TBZ or IMZ and never occurred alone. SOPP resistance in *P. digitatum* populations may have been perpetuated because it is selected for based on the other fungicide resistance present in these isolates.

Our work corroborates early studies that reported the presence of fungicide-resistant isolates of *P. digitatum* and *P. italicum* within packing houses and not in groves (Eckert and Wild, 1983; El-Goorani et al., 1984; Holmes and Eckert, 1995, 1999). Harding (1972) and Kuramoto (1976) reported that a few TBZ-resistant strains of *P. digitatum* could be found naturally in groves, even if they had never been exposed to the fungicide. This differs from the situation with post-harvest blue mould of pomes, caused by *P. expansum*, where resistant isolates occur primarily in packing houses (Spotts and Cervantes, 1986; Baraldi et al., 2003) but also in orchards (Li and Xiao, 2005), although fungicides to control it are not applied there. A significant difference between citrus and pome management is the handling of harvest bins. Harvest bins used in citrus industry are usually not used for fruit storage and rigorously cleaned between harvests, so conidia of resistant isolates are not introduced into citrus groves. Conversely, those used for pomes are both used for long storage and often not cleaned between harvests, so conidia of the resistant isolates can be introduced into orchards (Sholberg, 2005). Therefore, the practice of rigorous bin sanitation should be practiced. If the same fungicides are used in groves as in packing houses, resistance would likely develop soon afterward. Kuramoto (1976) reported in Japan, where benzimidazole fungicides were applied only before harvest in groves, that most isolates from decayed fruit collected in groves and packing houses were resistant to TBZ. In California now, two fungicides are proposed for use in groves, thiophanate methyl (Smilanick et al., 2006) and azoxystrobin.

Although the efficacy of TBZ and IMZ has declined commercially both remain in common use and are effective, particularly where the development of resistance is closely monitored.

Baseline sensitivity is the response of previously unexposed fungal individuals or populations to a fungicide (Russell, 2004). The baseline EC_{50} concentrations of IMZ, TBZ, and SOPP of *P. digitatum* isolates we observed were similar to those reported by others. The EC_{50} values of

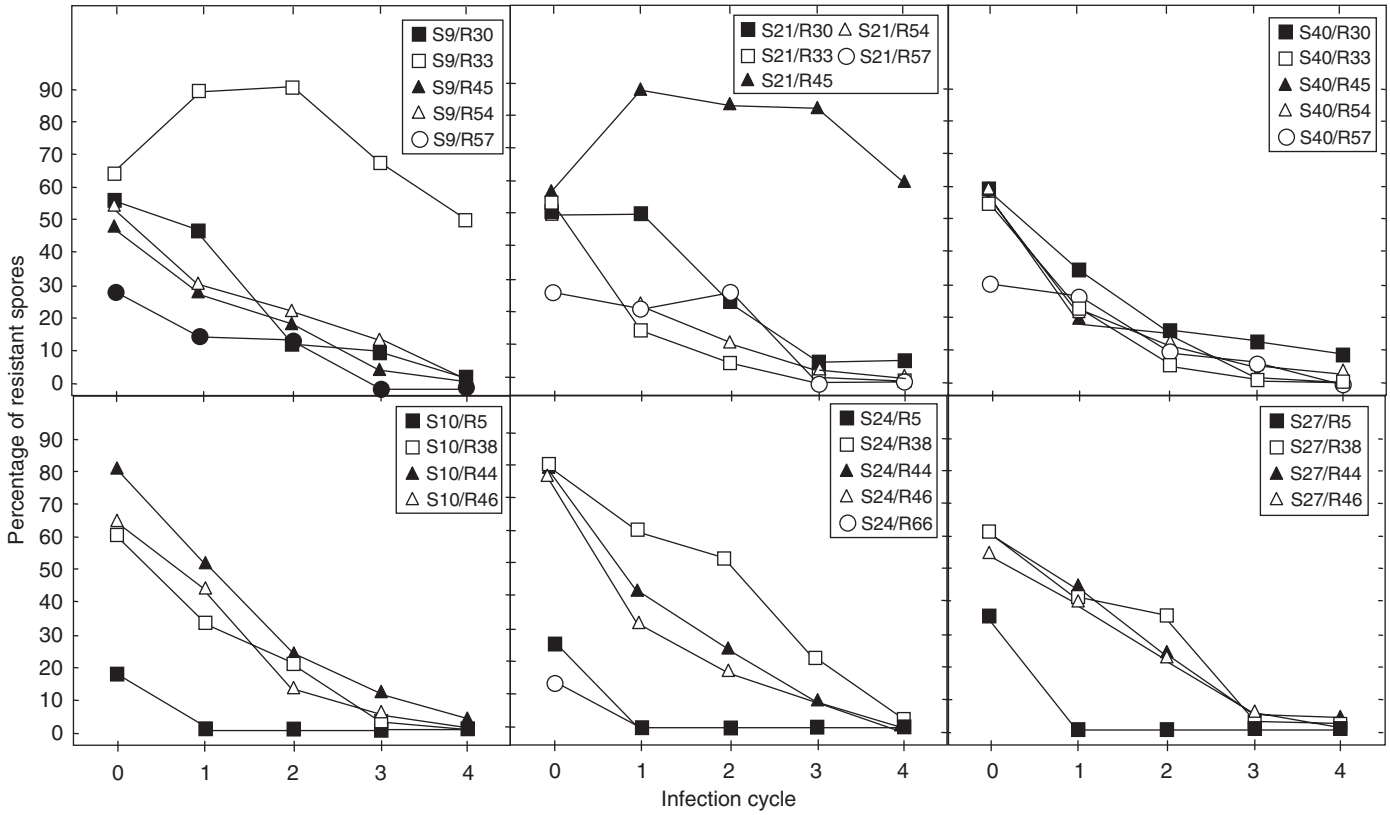

Fig. 2. Percentage of imazalil-resistant conidia of *Penicillium digitatum* initially and after four sequential cycles of infection of orange fruit. The initial inoculum consisted of 10^6 conidia ml^{-1} of sensitive (S) and resistant (R) isolates mixed in a 1:1 ratio. A total of 28 unique combinations of S and R isolates were tested. After each 7 d cycle, conidia were collected from the infected fruit, used as inoculum to initiate the following cycle, and the percentage of imazalil-resistant conidia was enumerated on potato-dextrose agar alone or amended with 0.1 μg ml^{-1} imazalil. Imazalil-sensitive ($n = 6$) and imazalil-resistant ($n = 10$) isolates were a geographically diverse sample of the isolates collected in California (Table 3). All of the S isolates were from infected fruit found in groves or under isolated trees, while R isolates were from infected fruit in packing houses.

isolates resistant to these fungicides were also similar to the values in prior reports.

The EC_{50} ($\pm$SD) of *P. digitatum* isolates we classified as sensitive ($n = 66$) to IMZ was 0.044 ± 0.023 μg ml^{-1} (Table 1). Values reported by others are similar (Wild, 1994), but some variation is to be expected because, in addition to differences in inoculation and media, inoculum age (Holmes and Eckert, 1999) and pH (Holmes and Eckert, 1999; Smilanick et al., 2005) greatly influence the sensitivity of *P. digitatum* to this fungicide. The baseline sensitivity of *P. digitatum* in Florida to IMZ was 0.027–0.146 μg ml^{-1} (Brown, 1989) and 0.026 ± 0.003 μg ml^{-1} in California (Holmes and Eckert, 1999). In our work, the EC_{50} of those resistant ($n = 100$) to IMZ was 1.45 ± 0.58 μg ml^{-1}. The EC_{50} of *P. digitatum* isolates resistant to IMZ was 1.5 μg ml^{-1}, with a standard deviation of 0.5, among Australian isolates (Wild, 1994), and 0.92–1.73 μg ml^{-1} among those in California (Holmes and Eckert, 1999).

The EC_{50} ($\pm$SD) of *P. digitatum* isolates we classified as sensitive ($n = 113$) to TBZ was 0.1 ± 0.1 μg ml^{-1}. The EC_{50} of those resistant ($n = 53$) to TBZ was 17.7 ± 4.8 μg ml^{-1}. In prior work, the baseline sensitivity of *P. digitatum* to TBZ was 0.052–0.058 μg ml^{-1} (Seidel et al., 1990), 0.10–0.12 μg ml^{-1} (Tadeo et al., 1988), 0.15 μg ml^{-1} (Wild, 1980), and up to 0.6 μg ml^{-1} (Kitagawa and Tani, 1984). Bus (1992) found that the mean EC_{50} level of the *P. digitatum* isolates resistant to TBZ collected from many parts of the world was 15.8 μg ml^{-1}. The EC_{50} ($\pm$SD) of *P. digitatum* isolates we classified as sensitive ($n = 137$) to SOPP was 8.8 ± 1.7 μg ml^{-1}. The EC_{50} of those resistant ($n = 29$) to SOPP was 30.1 ± 9.1 μg ml^{-1}. Harding (1962) reported the EC_{50} values for the growth of *P. digitatum* isolates collected in California that were sensitive or resistant to SOPP were approximately 5 and 30 μg ml^{-1}, respectively.

The EC_{50} ($\pm$SD) of *P. digitatum* isolates we classified as sensitive ($n = 163$) to PYR was 0.30 ± 0.07 μg ml^{-1}. The EC_{50} of those resistant ($n = 3$) to PYR was 2.39 ± 0.2 μg ml^{-1}. The baseline EC_{50} concentrations of PYR to *P. digitatum* we observed were similar to those reported for other fungi. Sholberg et al. (2005) reported the sensitivity of *Penicillium* spp. isolates on apples to PYR varied greatly, from 0.050 to 0.566 μg ml^{-1}. Similarly, Li and Xiao (2005) also reported that baseline EC_{50} concentrations of PYR for *P. expansum* isolates were variable, from 0.52 to 2.05 μg ml^{-1}. Both of these studies characterized isolates collected before the fungicide was introduced. The EC_{50} concentrations of PYR within populations of

other fungi vary widely. For example, EC_{50} concentrations of PYR of sensitive isolates of *Botrytis cinerea* collected in Spain in 1992, before PYR was introduced, were 0.05–0.50 $\mu g\,ml^{-1}$ (Moyano et al., 2004), although Koller et al. (2005) found the EC_{50} values of *Venturia inaequalis* populations to PYR were distributed narrowly, from 0.12 to 0.30 $\mu g\,ml^{-1}$.

The behaviour of fungicide-resistant isolates in competition experiments with sensitive isolates may provide some information to interpret their persistence commercially. For example, *sec*-butyl amine resistance was first found in *P. digitatum* populations shortly after its introduction (Harding, 1976). Resistance to this material developed among stored lemons quite rapidly, after 2 or 3 months (Houck, 1977). Isolates with *sec*-butyl amine resistance in mixtures with sensitive isolates inoculated into fruit or on growth media did not decline in the absence of the fungicide (Smilanick and Eckert, 1986). After several years of commercial use, its efficacy declined commercially to very low levels and this material is no longer registered for use on citrus fruit. Conversely, TBZ resistance developed among stored lemons more slowly, after 4 or 5 months (Houck, 1977), and isolates with benzimidazole resistance in mixtures with sensitive isolates inoculated into fruit or on growth media declined in the absence of the fungicide (Eckert and Wild, 1983; Seidel et al., 1990).

Our work characterizing IMZ-resistant isolates of *P. digitatum* corroborates that of others in many aspects. Similar to Holmes and Eckert (1995), we found the cost of the acquisition of IMZ resistance is primarily a loss in fitness of IMZ-resistant isolates in competition with sensitive biotypes; in most mixtures of resistant isolates with sensitive isolates, the proportion of IMZ-resistant conidia declined after several cycles if the fungicide was not present. Van Gestel (1988), in a study of IMZ-sensitive and less sensitive strains of *P.digitatum* competition with each other concluded that the less sensitive strains were not able to compete fully against sensitive ones, and that in lesions caused by less-sensitive strains alone, there was a shift-back to IMZ sensitivity in the conidia produced. De Waard et al. (1982) studied the persistence on oranges of *Penicillium italicum* isolates with a high degree of resistance to fenarimol, an ergosterol biosynthesis inhibitor like IMZ. In their competition experiments with mixed inocula of fenarimol-sensitive and -resistant isolates, the resistant isolates could still be isolated after five successive infection cycles on fenarimol-free oranges. Nevertheless, the proportion of resistant conidia in the successive populations gradually decreased.

In our work, with isolates collected 10 or more years later from about the same areas as Holmes and Eckert (1995), we found the resistant isolates were typically more persistent than they did in competition with sensitive isolates. Resistant conidia were generally present up to three or four cycles in our work, while most were absent after one or two cycles in their work, which is evidence that the fitness of IMZ-resistant isolates may have improved. However, we both observed that in some combinations of fungicide-sensitive and -resistant isolates the proportion of IMZ-resistant isolates did not decline. Interestingly, we both found that in cases where the IMZ-resistant isolate persisted in combination with a sensitive isolate, it was also resistant to other fungicides (TBZ and SOPP). Similarly to Holmes and Eckert (1995), we found lesion expansion rates alone did not predict competitive ability during repeated infection cycles. Unlike their conclusion that the acquisition of IMZ resistance did not alter the pathogen, we found it was associated with small but significant reductions in colony size and lesion sizes, and the colony colour of resistant isolates was slightly but significantly lighter in colour. The colour of these colonies cannot be used to detect them in practice, because the alteration in colour was modest and the colonies darken rapidly as they age. The lighter colour may have been a result of the slower development of colonies by the fungicide-resistant isolates.

Periodic monitoring of packing house fungus populations provides advance warning of a developing resistance problem and could indicate which fungicide(s) is involved, and where the problem may be originating in the packing house (Houck, 1977). Recently, new technologies to monitor resistant isolates of *P. digitatum* have been developed, including an air-sampling method combined with a spiral-gradient dilution plate to rapidly quantify resistance to a number of fungicides (Förster et al., 2004; Soto-Estrada et al., 2004), and a PCR-based method to detect IMZ resistance in 5–6 h (Hamamoto et al., 2001).

When resistance to a fungicide is detected, an alternate fungicide with a different mode of action should be used and strict sanitation measures employed. PYR was only recently introduced as citrus post-harvest fungicide in California (Smilanick et al., 2006) and was not in use at the time our collections were made. The primary purpose for its introduction is to control *P. digitatum* isolates resistant to the other fungicides. We found no PYR-resistant isolates in packing houses, although we did find three resistant isolates from relatively isolated groves in Butte County in the Sacramento Valley. Kanetis (2005) selected *P. digitatum* isolates after repeated exposures to PYR that were less sensitive to the fungicide and poorly controlled by recommended rates of the fungicide. These findings indicate the propensity for the development of resistance to PYR may be high. Thus, the introduction of PYR into packing houses should be done with actions to minimize and monitor the development of resistant isolates. These include its use with heat or sodium bicarbonate (Smilanick et al., 2006), in mixtures or in alternations with other fungicides. Prusky et al. (1985) reported the best strategy to control TBZ-resistant isolates of *P. expansum* on pome fruits in storage was a heterogeneous treatment, where a portion of the fruit within a storage room was treated with a mixture of TBZ and a newly introduced fungicide with a different mode of action, and some were treated only with the new fungicide. This strategy both reduced the disease most effectively and resulted in the most rapid decline in

the proportion of the spore population resistant to TBZ, compared to strategies where all the fruit were treated with a mixture of both fungicides.

Measures to improve sanitation in packing houses can reduce the proliferation of resistant isolates (Hall and Bice, 1977; Bancroft et al., 1984; Gardner et al., 1986; Goodwine, 2005). A practice that contributes to the spread of fungicide-resistant inoculum is the manual removal of diseased, fungicide-treated fruit from storage boxes or cartons, particularly when this is done inside packing houses. Commonly needed after long storage or if sale of fruit in cartons is delayed, this operation should be isolated as much as is feasible from the packing house to minimize the spread of the fungicide-resistant inoculum present on these fruits.

This work describes some aspects of fungicide resistance in *P. digitatum* and why it has been such a severe and prevalent problem within citrus packing houses. No fungicide-resistant isolates were present in groves, with the exception of PYR. Although most fungicide-resistant isolates exhibited slightly altered growth and reduced competitive ability, when some were paired with sensitive isolates they persisted for relatively long periods. This suggests there may be selection for improved levels of fitness and that their competitive ability and persistence may increase in the future.

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